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Both GacS-regulated lipopeptides and the type three secretion system contribute to *Pseudomonas cichorii* induced necrosis in lettuce and chicory

Chien-Jui Huang [a,b,*], Ellen Pauwelyn [a,c], Marc Ongena [d], Peter Bleyaert [c], Monica Höfte [a,**]

[a] Department of Plants and Crops, Laboratory of Phytopathology, Ghent University, Coupure Links, 653, 9000 Ghent, Belgium
[b] Department of Plant Medicine, National Chiayi University, No. 300, Syuefu Rd., 600355, Chiayi, Taiwan, Republic of China
[c] Inagro Vzw, Ieperseweg 87, 8800, Rumbeke, Belgium
[d] Microbial Processes and Interactions Laboratory, Terra Teaching and Research Center, Gembloux Agro-Bio Tech, University of Liège, Gembloux, Belgium

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ABSTRACT

Pseudomonas cichorii SF1-54, the causal agent of lettuce midrib rot disease, produces lipopeptides cichofactins and cichopeptins which are important virulence factors. The GacS/GacA two-component system is well known to regulate production of lipopeptides in pseudomonads. Additionally, the functions of the type three secretion system (T3SS) in *P. cichorii*-plant interactions are not clarified. In this study, we investigated the role of the GacS-regulated lipopeptides and the T3SS in pathogenicity of *P. cichorii* SF1-54 on two host plants, chicory and lettuce, by constructing mutants in *hrpL*, which encodes the key sigma factor to control T3SS expression, and *gacS*. Compared with the wildtype, the *hrpL* mutant produced lipopeptides at a similar level but the *gacS* mutant was strongly impaired in lipopeptide production. The mutant deficient in *hrpL* did not significantly differ from the wildtype in virulence on chicory and lettuce. The *gacS* mutant exhibited significantly less symptoms on both host plants compared to the wildtype and the *hrpL* mutant. Intriguingly, the *gacS hrpL*-double mutant no longer produced lipopeptides, lost virulence and showed impaired colonization on chicory, but was still weakly virulent on lettuce. Thus, contribution of both the GacS-regulated lipopeptides and T3SS to virulence of *P. cichorii* SF1-54 is host plant dependent.

1. Introduction

Two-component systems are used by bacteria to sense and to respond to extra- and intracellular environments. These systems regulate expression of many genes, especially secondary metabolites, and help bacteria to adapt to their environment (reviewed in Laub and Goulian [1]). One of the well-studied bacterial two component systems, especially in pseudomonads, is the GacS/GacA system. The sensor kinase (GacS) is autophosphorylated upon sensing environmental signal(s) and subsequently phosphorylates its cognate response regulator (GacA), which activates the transcription of two or more small RNA (sRNA) encoding genes. These sRNAs sequester translational repressors such as RsmA. RsmA is an RNA binding protein that prevents translation of mRNAs encoding secondary metabolites [2–4]. By alleviating translational repression, the GacS/GacA two-component system positively controls production of secondary metabolites including phytotoxic compounds [2,3]. In fact, *gacS*, originally named *lemA* (lesion manifestation), was first identified in the bean pathogen *Pseudomonas syringae* pv. *syringae* B728a, and the *gacS* mutant lost its virulence on bean [5]. Although phytotoxin production is generally under its regulation [6–8], the GacS/GacA two-component system is not generally essential for lesion formation by plant-pathogenic pseudomonads [9]. For instance, the *gacS* mutant of *P. syringae* pv. *coronafaciens* still caused necrosis on oat leaves [9]. Moreover, the Gac/Rsm regulatory network varies widely among plant-pathogenic pseudomonads, and the requirement of the GacS/GacA system for expression of distinct phenotypes is pathovar- or species-specific [7,9–11].

Phytopathogenic bacteria must be able to enter the plant, to evade or suppress general antimicrobial defenses and acquire nutrients and water from their hosts to successfully colonize and grow within the host tissue and to cause disease. To achieve these goals, plant pathogenic bacteria may deploy an arsenal of virulence factors to modulate host cell processes [12]. One of these virulence factors is the type three secretion system (T3SS) by which bacteria inject effector proteins into the host

* Corresponding author. Department of Plants and Crops, Laboratory of Phytopathology, Ghent University, Coupure Links, 653, 9000 Ghent, Belgium.
** Corresponding author.
E-mail addresses: chienjui.huang@mail.ncyu.edu.tw (C.-J. Huang), monica.hofte@ugent.be (M. Höfte).

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cell. Many gram-negative plant pathogenic bacteria require this T3SS to cause disease on susceptible hosts and to elicit a hypersensitive response (HR) on resistant hosts [13]. Mutants defective in T3SS are usually unable to grow and to cause disease on susceptible hosts, indicating that the integrity of the T3SS is essential for pathogenicity [14]. Expression of the T3SS in *P. syringae* is regulated by HrpL, an alternative sigma factor belonging to the extracytoplasmic function (ECF) family [15,16].

Pseudomonas cichorii is a broad-host-range phytopathogenic bacterium belonging to phylogroup 11 in the *P. syringae* complex [17], which is increasing in importance. *P. cichorii* SPC9018 relies on the T3SS and iron acquisition to cause bacterial rot on celery, eggplant, okra, and sweet pepper, while T3SS and iron acquisition is not essential for pathogenicity on lettuce [18–21]. *P. cichorii* JBC1 requires the T3SS effectors AvrE1 and HopA1 for full virulence in cabbage and tomato [22,23]. However, our previous studies have shown that (cyclic) lipopeptides are important virulence factors for *P. cichorii* SF1-54, the causal agent of midrib rot disease of greenhouse-grown butterhead lettuces in Belgium [24–26]. *P. cichorii* SF1-54 can produce seven lipopeptide compounds including three classes of structurally related lipopeptides [25]. These seven lipopeptide compounds differ in antimicrobial spectra and in the ability to cause necrotic symptoms on chicory leaves. Phytotoxicity of the purified lipopeptide compounds were assayed, and only compounds G and F (further named cichopeptins A and B) are phytotoxic and able to cause necrotic symptoms on chicory. The other five compounds are antimicrobial compounds [25]. Two identified classes of lipopeptides, namely cichofactins and cichopeptins, synthesized by non-ribosomal peptide synthetases, significantly contribute to virulence of *P. cichorii* SF1-54 on lettuce [25,27]. Cichofactins are linear lipopeptides of the Factin family [28] and are involved in surface motility and plant colonization [25], while cichopeptins are phytotoxic cyclic lipopeptides [27] belonging to the Peptin family [28]. *P. cichorii* strains also produce a cyclic lipopeptide of the Mycin family which is most likely pseudomycin [28], but this has not been chemically confirmed.

The aim of this study was to explore the role of the GacS/GacA two-component system in lipopeptide production and virulence determination in *P. cichorii* SF1-54. Furthermore, we were also interested in understanding the function of T3SS in *P. cichorii*-host plant interactions. Thus, single and double mutants in *gacS* and *hrpL* of *P. cichorii* SF1-54 were constructed and characterized phenotypically. The requirement of GacS-regulated lipopeptides and T3SS for virulence of *P. cichorii* was tested using pathogenicity assays on host plants that are either very susceptible to *P. cichorii* (lettuce) or disease tolerant (chicory). We show that both GacS-regulated lipopeptides and the HrpL-controlled T3SS can cause necrosis-like symptoms in lettuce and cichory. However, their coordinated contributions to pathogenicity/virulence of *P. cichorii* SF1-54 are host plant dependent.

2. Materials and methods

2.1. Microorganisms and culture conditions

Microorganisms used in this study are listed in Table 1. *P. cichorii* SF1-54Nal^R and its mutants were routinely grown on Pseudomonas Agar F (PAF, Difco, Erembodegem, Belgium) or SRM$_{AF}$ medium for lipopeptide production [29]. *Rhodotorula mucilaginosa* MUCL 30397,

Table 1
Strains, plasmids and primers used in this study.

Strain, plasmid or primer	Genotype or description[a]	Source/Reference
Strains		
Pseudomonas cichorii		
SF1-54	Natural isolates from infected greenhouse butterhead lettuce in Belgium	Cottyn et al., 2011
SF1-54Nal^R	A spontaneous nalidixic acid resistant mutant of *P. cichorii* SF1-54	Pauwelyn et al., 2013
SF1-54Δ*gacS*	A *gacS*-deletion mutant of SF1-54Nal^R	Pauwelyn et al., 2013
SF1-54Δ*hrpL*	A *hrpL*-deletion mutant	This study
SF1-54Δ*gacS*Δ*hrpL*	A *gacS* and *hrpL*-deletion mutant	This study
Escherichia coli S17-1	*thi pro hsdR rec*A::RP4-2-Tc^R::Mu Km^R::Tn7(λ*pir*), Sm^R	Simon et al., 1983
Bacillus megaterium LMG 7127	An indicator strain for lipopeptide	
Rhodolotula mucilaginosa MUCL 30397	An indicator strain for lipopeptide	
Geotrichum candidum MUCL 28959	An indicator strain for lipopeptide	
Saccharomyces cerivisiae InvSc1	*MATa/MATα leu2/leu2 trp1-289/trp1-289 ura3-52/ura3-52 his3-Δ1/his3-Δ1*	Invitrogen
Plasmids		
pMQ30	7.6 kb mobilizable suicide vector used for gene replacements in *Pseudomonas* spp.; *Sac*B, URA3, Gm^R	Shanks et al., 2006
pMQ30-*gacS*	pMQ30 containing a 1 kb fragment upstream and downstream of *P. cichorii* SF1-54 *gacS* gene to obtain the *gacS* deletion	This study
pMQ30-*hrpL*	pMQ30 containing a 1 kb fragment upstream and downstream of *P. cichorii* SF1-54 *hrpL* gene to obtain the *hrpL* deletion	This study
pJEL5771	Contains *gacS* of *Pseudomonas fluorescens* Pf-5, Tc^R	Corbell and Loper, 1995
pBBR1MCS-5	Broad-host-range cloning vector, Gm^r	Kovach et al., 1995
pHrpL	pBBR1MCS-5 containing the intact *hrpL* gene of *P. cichorii* SF1-54 and its native promoter	This study
Primers (5'→3')		
gacS-F1	*GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTG*CCAAACTGGGTATAAAAGGC	This study
gacS-R1	*GGGATTTCATGGGCCGTGAC*CGTTGATGATGCTCAGCAGG	This study
gacS-F2	*CCTGCTGAGCATCATCAACGG*TCACGGCCCATGAAATCCC	This study
gacS-R2	*CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGAT*GACGGCCAGTCGGGTAATGG	This study
hrpLF1long	*GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTG*ACCAGCCTATCGTCTGATCG	This study
hrpLR1long	*ACAAGTATCTGGGCACCTCCC*CATGCGTACCGTCATTCAAC	This study
hrpLF2long	*GTTGAATGACGGTACGCATG*GGAGGTGCCCAGATACTTGT	This study
hrpLR2long	*CCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGAT*GATATGCGCACCACCTGTT	This study
hrpLctrlF	AAACGCTGGATTTGAGCTGT	This study
hrpLctrlR	AAAGCAGCGAGAAAAAGCTG	This study
hrpL-*Sal*I	CGGTCGACTGAACTTCATCGCCACTTCT	This study
hrpL-*Pst*I	CGCTGCAGCGCTTGTAGAGGTTGACGTA	This study

[a] Primer extensions are in italic font. Tc^r, Km^r, Sm^r, and Gm^r indicate resistant to tetracycline, kanamycin, streptomycin, and gentamycin, respectively.

Geotrichum candidum MUCL 28959 and *Bacillus megaterium* LMG 7127 were used as indicator organisms for bioassays of antimicrobial activities [25]. *B. megaterium* and *Escherichia coli* strains were grown on Luria-Bertani (LB) medium [30] at 28 °C and 37 °C, respectively. *G. candidum* and *R. mucilaginosa* were cultured on PDA at 28 °C. *Saccharomyces cerevisiae* was grown on yeast-extract-peptone-dextrose medium at 30 °C [31]. All bacterial strains and *R. mucilaginosa* were maintained at −80 °C in LB broth with 20 % glycerol. Antibiotics were added to the growth media (if required) at the following concentrations: 100 μg/mL gentamycin, 10 μg/mL nalidixic acid, 10 μg/mL tetracycline.

2.2. Construction of the gene specific deletion mutants of *P. cichorii* SF1-54 and complementation

A gene specific-deletion mutant of *P. cichorii* SF1-54Nal^R was constructed by means of an *in vivo* cloning technique with the yeast *S. cerevisiae* InvSc1 [31,32]. To construct deletion plasmid pMQ30-Δ*gacS*, primers GacS-F1 and GacS-R1 were used to amplify the upstream fragment and primers GacS-F2 and GacS-R2 for the downstream fragment (Table 1). For deletion of the *hrpL* gene, plasmid pMQ30-Δ*hrpL* was constructed with primer pairs HrpL-F1/HrpL-R1 and HrpL-F2/HrpL-R2 for amplification of the upstream and downstream fragments, respectively (Table 1). A *gacS*-deletion mutant and a *hrpL*-deletion mutant contained 900 bp deletion in *gacS* and 201 bp deletion in *hrpL*, respectively. A *gacS hrpL* double mutant was constructed as well. Deletion of *gacS* or *hrpL*-fragment was confirmed by PCR and the mutant was characterized phenotypically.

To complement the *hrpL*-deletion mutant, a 1.1-kb fragment containing *hrpL* with 400 bp upstream and 187 bp downstream was amplified by PCR with primers HrpL-*Sal*I and HrpL-*Pst*I. The amplified fragment was cloned into *Sal*I- and *Pst*I sites of pBBR1MCS-5 [33]. The obtained plasmid pHrpL was introduced into the mutants deficient in HrpL by conjugation for complementation. On the other hand, plasmid pJEL5771 [34], carrying *gacS* of *P. protegens* Pf-5, was used for complementation of the *gacS* mutation.

2.3. Phenotypic characterization of *P. cichorii* strains

2.3.1. Phytotoxicity

Phytotoxic activity was tested on chicory or lettuce leaves by injection of 300 μL bacterial suspension [1.0 × 10^8 CFU/mL in 50 mM phosphate buffer (PB, pH 7.0)] or culture filtrate with a syringe through the abaxial surface. The injected leaves were incubated at 100 % humidity and 25 °C for 24 h and appearance of necrotic lesions was scored.

2.3.2. Antimicrobial activity

Antimicrobial activity was tested against *B. megaterium, G. candidum* and *R. pilimanae* using the method described by Pauwelyn et al. [25]. Briefly, bacterial suspensions of *P. cichorii* strains (1 × 10^8 CFU/mL) were spotted on PDA plates in five replicates, allowing growth at 25 °C for 5 days. The areas of colony growth were marked and the colonies removed with a sterile swab. The plates were exposed to chloroform vapors for 20 min to kill the remaining bacterial cells, followed by dissipation of chloroform vapors. The plate was then sprayed with a spore/cell suspension at a concentration about 10^6 cells or spores/ml of the indicator microorganism. After 24–48 h incubation at 28 °C, inhibition of the indicator microorganism was scored.

2.3.3. Swarming assay

Surface motility of *P. cichorii* SF1-54Nal^R and its mutants was assayed on soft LB plates (0.5 % agar). Bacteria of a fully grown colony on PAF-medium were applied in the centre of the soft agar plates with a sterile toothpick and the plates were subsequently incubated at 28 °C up to 72 h.

2.3.4. Lipopeptide production

A bacterial suspension of *P. cichorii* was added in SRM_{AF} medium (25 ml in a 100-ml flask) to a final concentration of 1 × 10^8 CFU/mL. Bacterial cultures were incubated at room temperature for 6 days without shaking. After six-day incubation, culture supernatants were collected and filtrated through 0.22-μm filters. Cell-free culture filtrates were used for phytotoxicity assay. and quantification of lipopeptide production by UPLC-ESI-MS analysis.

2.3.5. Pyoverdine measurement

Production of pyoverdine by *P. cichorii* SF1-54Nal^R and its mutants was estimated spectrophotometrically by measuring absorbance at 405 nm [35,36]. *P. cichorii* strains were cultured in King's B medium (Difco) at 28 °C for 3 days. The absorbance was normalized for differences in cell density.

2.4. Pathogenicity assay

Pathogenicity of *P. cichorii* strains was tested at Inagro, Rumbeke, Belgium, according to the methods described in our previous studies [25,26]. Butterhead lettuce plants cv. Hofnar (Rijk Zwaan, De Lier, the Netherlands) at head formation stage were inoculated with *P. cichorii* strains. Tap water without bacteria was used as the un-inoculated treatment. Each treatment was assayed in three replicates with 24 plants per replicate. At harvest, disease severity was assessed by giving each plant a score ranging from 0 to 4, where 0 = healthy plant, 1 = little black spots on the leaf periphery of the inner crop leaves, 2 = infection of small side ribs or black spots or stripes on the midrib, 3 = one, two or three rotten midribs, and 4 = four or more rotten midribs. Disease score of all plants was calculated as previously described [25,26]. All data were statistically analyzed using the software package SPSS 21.0 for Windows. As the data did not meet the conditions of normality and homogeneity of variance, non-parametric Kruskal-Wallis and Mann-Whitney comparisons ($P = 0.05$) were performed.

2.5. Population dynamics in planta

To test the ability of *P. cichorii* SF1-54Nal^R and its mutants to grow in lettuce midribs, the midrib of the inner lettuce leaves was injected with 1.0 ml bacterial suspension (1 × 10^5 CFU/ml). To test bacterial growth in chicory leaves, 300 μl of bacterial suspension (1 × 10^8 CFU/ml) was injected in the middle of each chicory leaf. The injected leaves were incubated at 100 % humidity at 25 °C. Nine leaf discs (1-cm diameter) were excised from the inoculated area of three leaves at 0, 1, 2, and 3 days post inoculation and macerated in 7.0 mL sterile 0.05 M potassium phosphate buffer (PB, pH 7.0). Tenfold serial dilutions from the macerate were prepared with PB and 100 μl aliquots of each dilution were spread on three PAF plates with 50 μg/ml nalidixic acid. Colonies were counted 2 days after incubation at 28 °C. The experiment was repeated twice. All data were statistically analyzed using the software package SPSS 21.0 for Windows. As the data met the conditions of normality and homogeneity of variance, Fisher's Least Significant Difference was performed for comparisons ($P = 0.05$).

3. Results

3.1. Sequence analysis of gacS/gacA genes and the hrp/hrc cluster of *P. cichorii* SF1-54

By mining the genome of *P. cichorii* SF1-54, we identified the genes encoding the GacS/GacA two-component system and the Hrp/Hrc proteins involved in T3SS. By Blast analysis, identical *gacS* and *gacA* were identified in the genome of *P. cichorii* JBC1 [37]. GacS (KP692744) and GacA (KP692743) of *P. cichorii* SF1-54 are similar to their protein homologs in other plant-associated pseudomonads (Supplementary Table S1). The gene organization of the *hrp/hrc* cluster (OQ744173) of

P. cichorii SF1-54 was compared with the previously characterized *hrp/hrc* clusters of *P. cichorii* 83-1 (Araki et al., 2006) and *P. cichorii* SPC9018 (Hojo et al., 2008) and with the genome of *P. cichorii* JBC1 [37]. Like in the other strains, the *hrp/hrc* cluster of *P. cichorii* SF1-54 forms a single pathogenicity island (S-PAI) without effector loci at both 5′- and 3′-ends. The *hrp/hrc* cluster of *P. cichorii* SF1-54 is 49.664 kb in size and contains 50 open reading frames (ORF). The *hrp/hrc* genes of *P. cichorii* SF1-54 are genetically organized in the same way as in *P. cichorii* strains 83-1 [38], SPC9018 [18], and JBC1 [37], and the subdivision into four regions (A, B, C and D), which was described by Hojo et al. [18], is conserved. The ORF initially identified as C13 in strain SPC9018 is variable among the *P. cichorii* strains (Supplementary Table S2). ORF C13 of SPC9018 encodes a pirin/FND-dependent NADH azoreductor fusion protein but this ORF does not exist in the PAI of *P. cichorii* 83-1 [18]. Moreover, the genomes of SF1-54 and JBC1 show two ORFs (designated ORF C13a and ORF C13b) encoding a

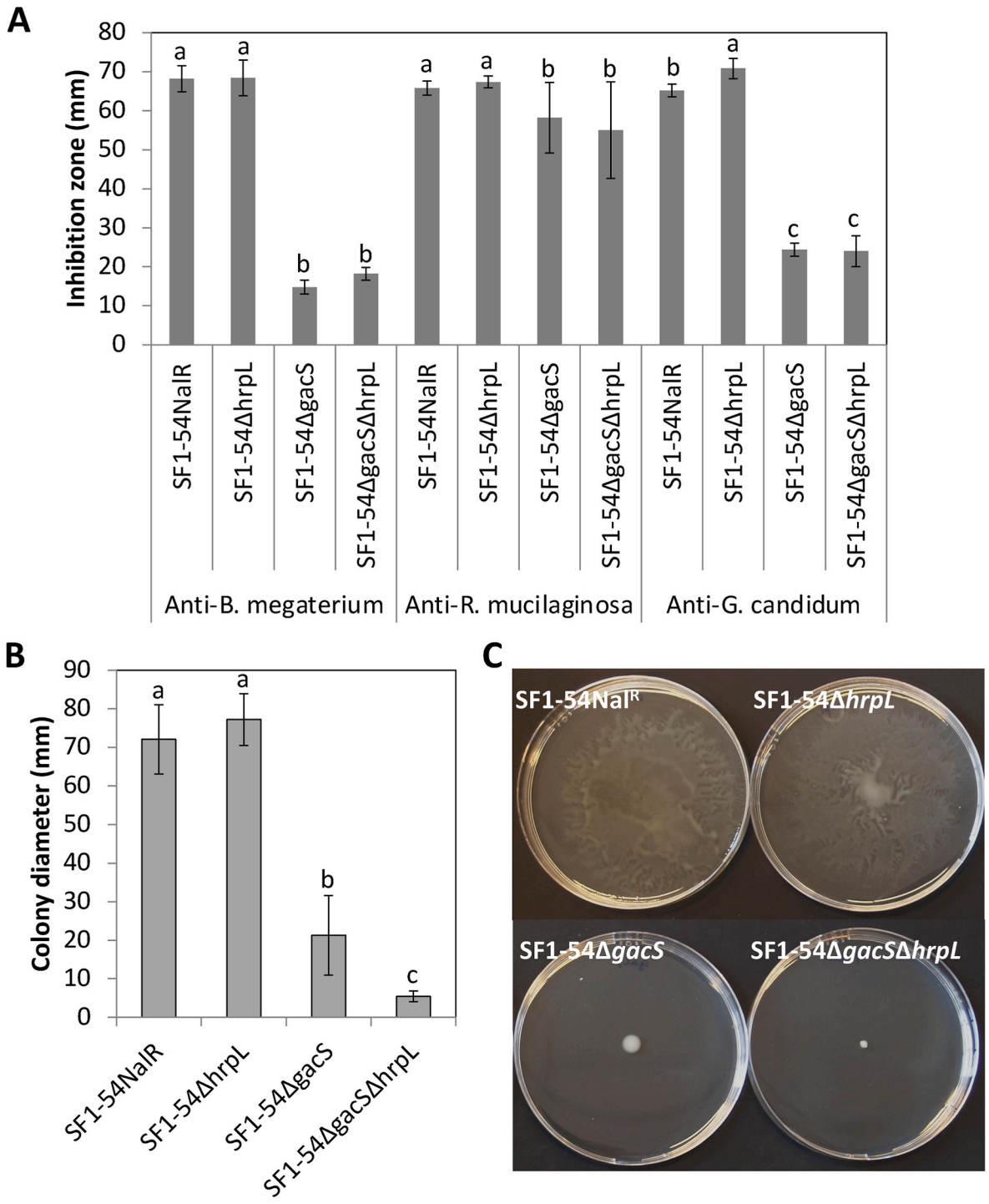

Fig. 1. Phenotypic characterization of *Pseudomonas cichorii* SF1-54Nal^R and its mutants deficient in GacS or HrpL. (A) Antimicrobial activities of *P. cichorii* SF1-54Nal^R and its mutants against three indicator microorganisms. Values represent the mean ± standard deviation of five repetitions. (B) Swarming behavior of *P. cichorii* strains. Values represent the mean ± standard deviation of two experiments with five repetitions per experiment. Bars indicated with the same letter are not statistically different based on nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$). (C) Swarming behavior of *P. cichorii* SF1-54 and its mutants on Luria-Bertani soft agar (0.5 % [wt/vol]) plates.

FND-dependent NADH azoreductor (ORF C13a) and a pirin (ORF C13b), respectively, as shown in Supplementary Table S2. In addition to the ORFs, the consensus sequence of the *hrp*-box, GGAACC-N15/16-CCACNNA [39], preceeds the same *hrp* genes as in *P. cichorii* SPC9018, but the position of the genes to their preceding *hrp*-box differs among the *P. cichorii* strains, ranging from a few nucleotides to 100 nucleotides (data not shown).

Because no effector loci were found at both 5′- and 3′-ends of the *hrp/hrc* cluster of *P. cichorii* SF1-54, we tried to identify possible effector genes by BlastP analysis with amino acid sequences of already known effectors (Database-Hops, http://www.pseudomonas-syringae.org/). However, no putative effector-encoding genes including *hopA1* were found except for *avrE1* locating within the *hrp/hrc* cluster of *P. cichorii* SF1-54. Moreover, *hrpW* and *hrpZ*, which encode harpins, are also present in the *hrp/hrc* cluster of this strain.

3.2. *In vitro* biological role of the T3SS and the GacS/GacA two-component system in *P. cichorii* SF1-54

To investigate the importance of the GacS/GacA two-component system and the T3SS in pathogenicity for *P. cichorii* SF1-54, we constructed SF1-54Δ*gacS*, SF1-54Δ*hrpL* and SF1-54Δ*gacS*Δ*hrpL* non polar mutants by deleting about 900- and 201-bp fragments in *gacS* and *hrpL*.

When droplets of cell suspensions of *P. cichorii* SF1-54Nal^R and *P. cichorii* SF1-54Δ*hrpL* were dipped on parafilm, the droplets collapsed immediately. A cell suspension of *P. cichorii* SF1-54Δ*gacS* exhibited reduced drop-collapsing activity and *P. cichorii* SF1-54Δ*gacS*Δ*hrpL* did not cause spreading of the water on the parafilm surface (data not shown). *P. cichorii* SF1-54Nal^R and SF1-54Δ*hrpL* showed almost identical growth inhibitory activities against *B. megaterium*, *G. candidum*, and *R. mucilaginosa*, which are indicator microorganisms for the seven bioactive lipopeptides [25,27] (Fig. 1A). Mutants *P. cichorii* SF1-54Δ*gacS* and SF1-54Δ*gacS*Δ*hrpL* displayed similar but greatly decreased activities against *B. megaterium* and *G. candidum* (Fig. 1A). Growth inhibition of *R. mucilaginosa* by all four strains was similar although *P. cichorii* SF1-54Δ*gacS* and SF1-54Δ*gacS*Δ*hrpL* showed slightly reduced inhibition zones compared with SF1-54Nal^R and SF1-54Δ*hrpL*.

P. cichorii SF1-54Nal^R and its *hrpL* mutant both exhibited vigorous swarming motility on soft LB medium (0.5 % agar) within 24 h, while swarming motility was greatly reduced in the *gacS* mutant and completely abolished in the *gacS hrpL* double mutant (Fig. 1B and C).

The wild type strain and all mutants tested were able to cause necrotic symptoms on detached lettuce leaves whether by injection or spray inoculation although *P. cichorii* SF1-54Δ*gacS* and SF1-54Δ*gacS*Δ*hrpL* caused weaker symptoms than the other strains (Fig. 2A).

Induction of discoloration and necrosis on chicory leaves by injection with *P. cichorii* SF1-54Δ*hrpL* was similar to that of the parental strain *P. cichorii* SF1-54Nal^R (Fig. 2B). The *gacS* mutant induced less necrosis than the wild type strain, while the SF1-54Δ*gacS*Δ*hrpL* double mutant

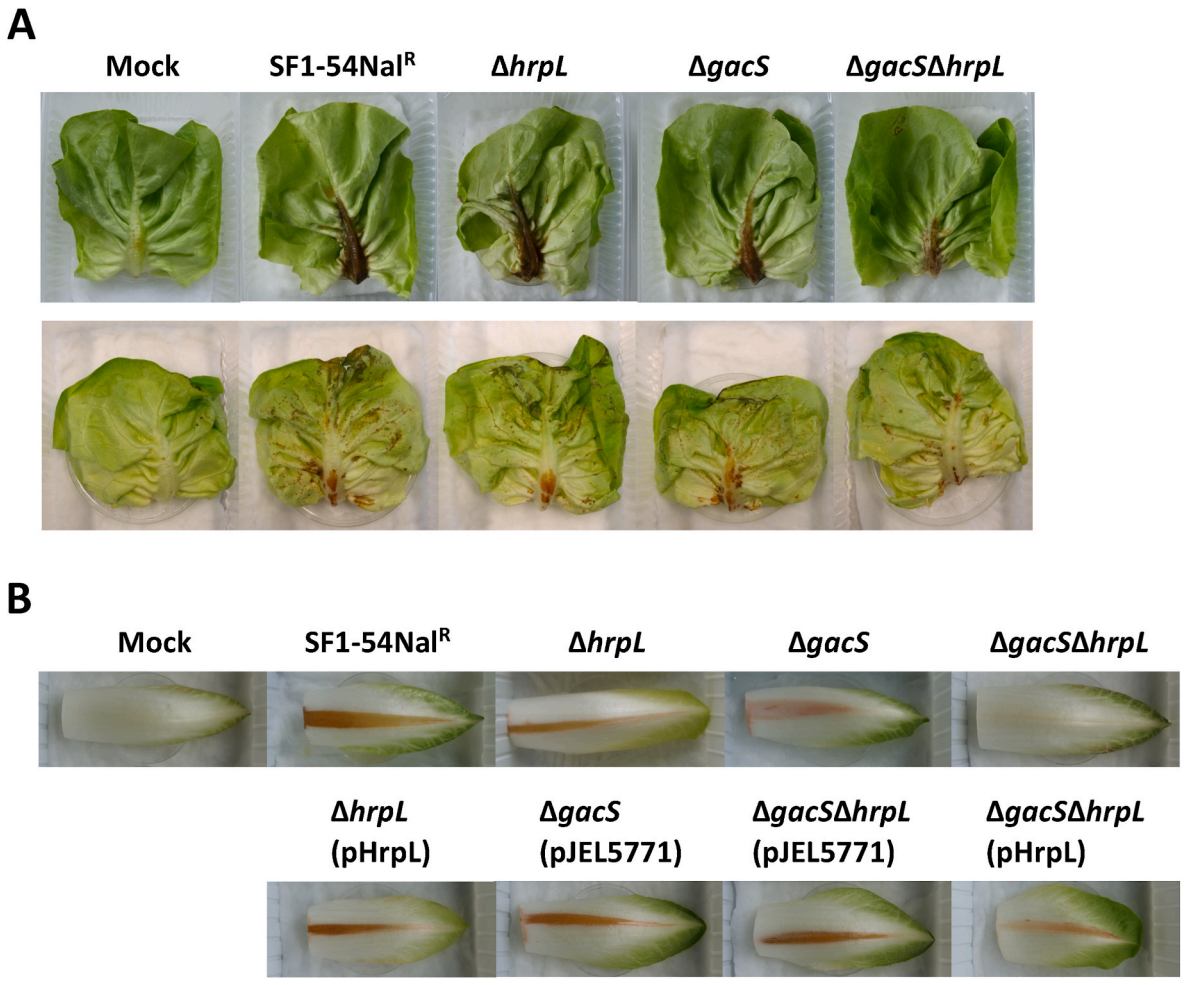

Fig. 2. Symptoms caused by *Pseudomonas cichorii* SF1-54Nal^R and its mutants deficient in GacS and/or HrpL on chicory and lettuce. (A) Lettuce leaves were injected with bacterial suspensions (upper panel), or inoculated by spraying bacterial suspensions (lower panel). (B) Chicory leaves were injected with bacterial suspensions of *P. cichorii* strains. *P. cichorii* strains are indicated. Photographs were taken at two days post inoculation (A) and one day post inoculation (B), respectively. Each treatment has been repeated three times and a picture representative of each treatment is shown.

completely lost the ability to cause discoloration and necrosis on chicory leaves (Fig. 2B).

P. cichorii SPC9018 requires iron acquisition ability to cause symptoms on chicory but not on lettuce [18–21]. Since *P. cichorii* produces pyoverdine to acquire iron from the environments [20,21], pyoverdine production by *P. cichorii* SF1-54Nal^R and its mutants was assayed. *P. cichorii* SF1-54Nal^R and its *hrpL* mutant both produced similar levels of pyoverdine, while production of pyoverdine was greatly but not completely reduced in the *gacS* mutant and the *gacS hrpL* double mutant (Supplementary Fig. S1).

3.3. GacS and HrpL are involved in regulation of lipopeptide production by P. cichorii

As revealed by UPLC-ESI-MS, both *P. cichorii* SF1-54Δ*hrpL* and the wild type strain produced all seven bioactive lipopeptide compounds (Fig. 3B). Compared with the parental strain, *P. cichorii* SF1-54Δ*hrpL* produced similar levels of cichofactins (indicated as compounds D and E in the chromatogram) and cichopeptins (compounds F and G), which are important virulence factors of *P. cichorii* SF1-54 (Fig. 3B and 4). *P. cichorii* SF1-54Δ*gacS* produced a greatly reduced level of cichofactins (compounds D and E) and cichopeptin A (compound G) and a residue level of cichopeptin B (compound F). Production of cichofactins and cichopeptins by *P. cichorii* SF1-54Δ*gacS*Δ*hrpL* was completely abolished (Fig. 3B and 4). A similar phenomenon was observed for the production of compound A and the pseudomycin-like compounds B and C with the mutant deficient in GacS producing lower amounts compared to the parental strain and the *hrpL* mutant. The results of *in vitro* bioactivities are in accordance to the results of UPLC-ESI-MS analysis.

We previously demonstrated that medium supplementation with glycine betaine can significantly enhance cichopeptin production by *P. cichorii* SF1-54 [27]. Hence, we were interested in whether or not the GacS/GacA two-component system was involved in the stimulation of cichopeptin production by glycine betaine. As expected, addition of glycine betaine significantly stimulated cichopeptin production (compounds F and G) by *P. cichorii* SF1-54Nal^R and its *hrpL* mutant (Fig. 5, Supplementary Fig. S2). Surprisingly, supplementation of glycine betaine slightly repressed cichofactin and cichopeptin production by *P. cichorii* SF1-54Δ*gacS*. Addition of glycine betaine had no effect on lipopeptide production by *P. cichorii* SF1-54Δ*gacS*Δ*hrpL*. To exclude the possibility that mutation in *gacS* inhibited uptake of glycine betaine by *P. cichorii*, we tested the utilization of glycine betaine by mutants SF1-54Δ*gacS* and SF1-54Δ*gacS*Δ*hrpL*. These two mutants were still able to utilize glycine betaine as the sole carbon source supplied to minimal medium (Supplementary Fig. S3).

Because *gacS* is conserved in pseudomonads, *P. cichorii* SF1-54Nal^R mutants deficient in GacS were functionally complemented by *gacS* of *Pseudomonas protegens* Pf-5 [34]. The complemented strains were restored in swarming motility (data not shown) and in their ability to cause dark brown necrosis on chicory (Fig. 2B). However, complementation of the *gacS hrpL* mutant with *hrpL* did not restore its virulence on chicory (Fig. 2B).

When chicory leaves were injected with culture filtrates of *P. cichorii* SF1-54Nal^R or *P. cichorii* SF1-54Δ*hrpL*, clear discoloration and necrosis on chicory leaves was observed. Compared to the parental strain, the culture filtrate of *P. cichorii* SF1-54Δ*gacS* showed reduced necrosis-

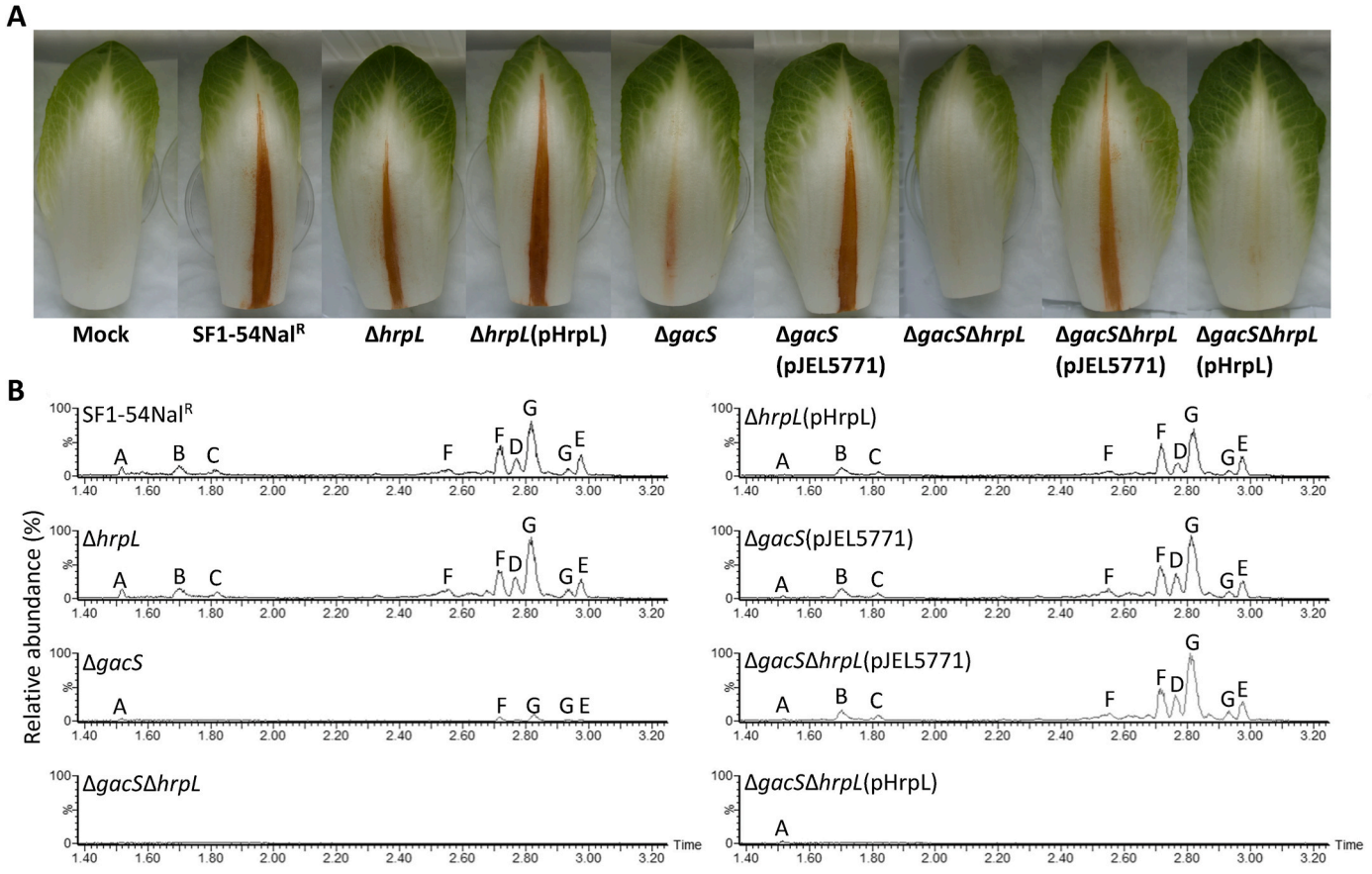

Fig. 3. (A) Symptoms caused by the culture filtrates of *P. cichorii* SF1-54Nal^R, its mutants deficient in GacS and/or HrpL and the mutants complemented for GacS (pJELS77) or HrpL (pHrp). Photographs were taken at two days post inoculation. (B) Chromatograms of the semipurified extracts of the culture filtrates obtained from *P. cichorii* strains grown in SRM_{AF} medium. Y axes of LC-MS traces are linked at the same scale for comparison of lipopeptide production. Seven bioactive compounds, which are indicated with a letter, in the semipurified culture extracts were identified by electrospray ionization mass spectrometry analysis. A, uncharacterized lipopeptidic compound. B and C, pseudomycin-like compounds. D and E, cichofactins. F and G, cichopeptins. *P. cichorii* strains are indicated.

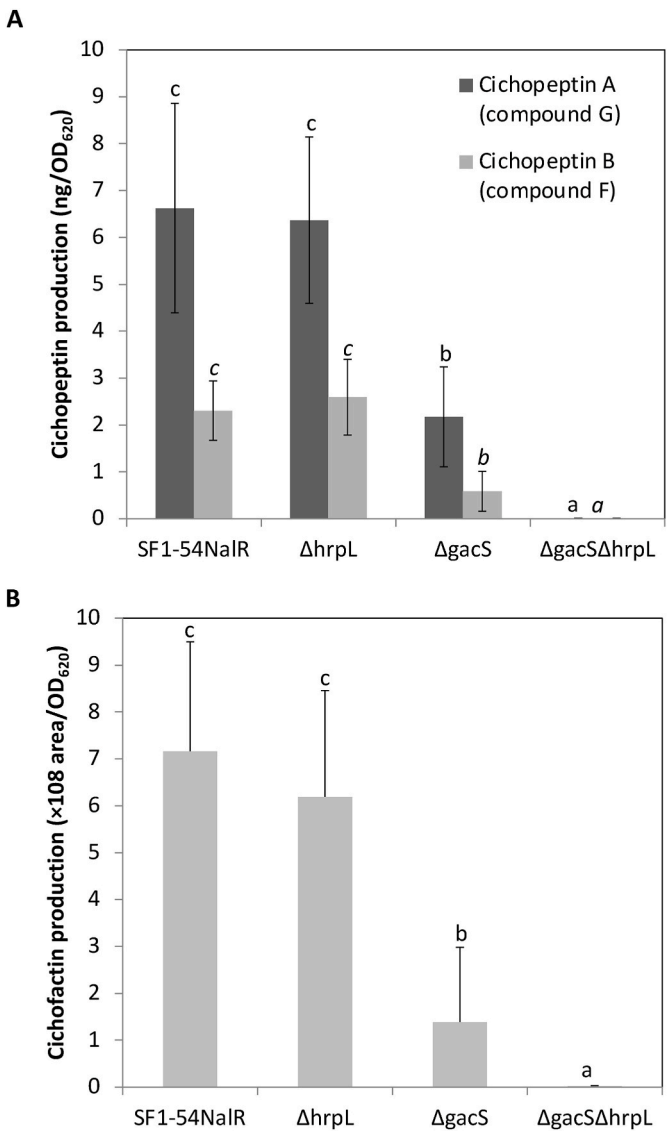

Fig. 4. Production of cichofactins and cichopeptins by *Pseudomonas cichorii* SF1-54Nal^R and its *gacS*, *hrpL*, and *gacS hrpL* mutants. Quantification of production of cichopeptins (A) and cichofactins (B) by *P. cichorii* based on UPLC-ESI-MS data. Values represent the mean ± standard deviation of five independent cultures from two experiments. Bars indicated with the same letter are not statistically different based on nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$).

inducing activity, while the culture filtrate of the *gacS hrpL* double mutant no longer caused necrosis (Fig. 3A). Furthermore, the culture filtrates of the *gacS*-complemented strains caused clear necrosis on chicory leaves (Fig. 3A) but plasmid-borne expression of *hrpL* did not complement the *gacS hrpL* mutant in production of phytotoxic compounds *in vitro* (Fig. 3B). UPLC-ESI-MS analysis confirmed that the *gacS*-complemented strains were restored in their production of lipopeptides (Fig. 3B), while complementation with *hrpL* did not restore the *gacS hrpL* mutant in production of lipopeptides to levels observed in the *gacS* mutant (Fig. 3B).

3.4. GacS-regulated lipopeptides and the T3SS contribute to virulence of *P. cichorii*

The involvement of the T3SS and/or the GacS/GacA two-component system on virulence of *P. cichorii* SF1-54 on lettuce was investigated under greenhouse conditions. Butterhead lettuce was spray-inoculated with *P. cichorii* SF1-54Nal^R and its mutants. The wild type and the *hrpL* mutant exhibited nearly identical virulence and caused severe rotten midribs at the same level (Fig. 6), while plants inoculated with mutants SF1-54Δ*gacS* and SF1-54Δ*gacS*Δ*hrpL* exhibited significantly less disease symptoms and severity ($P < 0.05$). Although the mutants SF1-54Δ*gacS* and SF1-54Δ*gacS*Δ*hrpL* did not significantly differ in induction of rotten midribs, SF1-54Δ*gacS*Δ*hrpL* caused a lower mean disease score than SF1-54Δ*gacS* (Fig. 6A).

When lettuce midribs were injected with SF1-54Nal^R or its mutants, all strains grew vigorously but reached different population densities in the inoculated midribs at 1, 2 and 3 days post inoculation (Fig. 7A). The population of the *hrpL* mutant was significantly lower than that of the wildtype at two and three days post inoculation. The *gacS* mutant grew *in planta* at a similar rate compared to the parental strain. *P. cichorii* SF1-54Δ*gacS*Δ*hrpL* also grew well *in planta* although the population of SF1-54Δ*gacS*Δ*hrpL* at one day post inoculation indicated a slight but significant delay in bacterial growth in lettuce midribs.

In addition, we also investigated the growth of all tested *P. cichorii* strains in chicory leaves. Chicory leaves were inoculated with a high-level inoculum of *P. cichorii* (1.0×10^8 CFU/mL, 1000 times higher than the inoculum for lettuce) because lower inoculum levels cannot cause symptoms on chicory. *P. cichorii* SF1-54Nal^R, the *gacS* mutant and the *hrpL* mutant grew well and reached similar population densities in the inoculated leaves at 1, 2 and 3 days post inoculation (Fig. 7B). However, populations of SF1-54Δ*gacS*Δ*hrpL* were significantly lower than populations of the other three strains tested and stabilized around 10^5-10^6 cfu/cm^2 in chicory leaves.

Furthermore, mutant SF1-54Δ*gacS*Δ*hrpL* complemented with *gacS* partly regained the ability to grow in chicory leaves (Fig. 8). But complementation with plasmid-borne *hrpL* did not restore the ability of the *gacS hrpL* double mutant to grow in chicory leaves (Fig. 8).

3.5. *P. cichorii* differs from *P. syringae* in the intergenic region between *hrpJ* and *hrpL*

Like *P. syringae*, complementation of SF1-54Δ*gacS*Δ*hrpL* with plasmid-borne *hrpL* was unsuccessful. That suggested that regulation of *hrpL* expression in *P. cichorii* might be similar to that in *P. syringae*. The intergenic region between *hrpJ* and *hrpL* of *P. cichorii* SF1-54 was compared with those of three phytopathogenic *P. syringae* reference strains. The sequence alignment shows *P. cichorii* SF1-54 clearly differs from *P. syringae* strains in size and sequence of the intergenic region (Supplementary Fig. S4). SF1-54 has a longer intergenic region (279 bp) than *P. syringae* (241–243 bp). Moreover, several gaps are shown in the alignment, differentiating *P. cichorii* from *P. syringae*. Like *P. syringae*, a *hrp* box and a HrpS binding site, GTGCCAAA [40], were found in the intergenic regions of *P. cichorii* regardless of sequence variation. HrpS is a bacterial enhancer binding protein and required for activation of *hrpL* transcription [15,40]. However, *P. cichorii* has an insertion of 8 bp in the HrpS binding site of the *hrpL* promoter, which results in a shorter sequence (GTGCCA) for recognition by HrpS.

4. Discussion

In this study, we investigated the role of the GacS/GacA two-component system and the T3SS in *P. cichorii*-host interactions by pathogenicity assay on lettuce and chicory. Both GacS-regulated lipopeptides and T3SS contributed to virulence of *P. cichorii* SF1-54 on lettuce, while the mutant deficient in GacS and HrpL completely lost virulence on chicory.

4.1. Sophisticated regulatory network of lipopeptide production by *P. cichorii* SF1-54

The GacS/GacA two-component system has been demonstrated to positively regulate production of secondary metabolites by

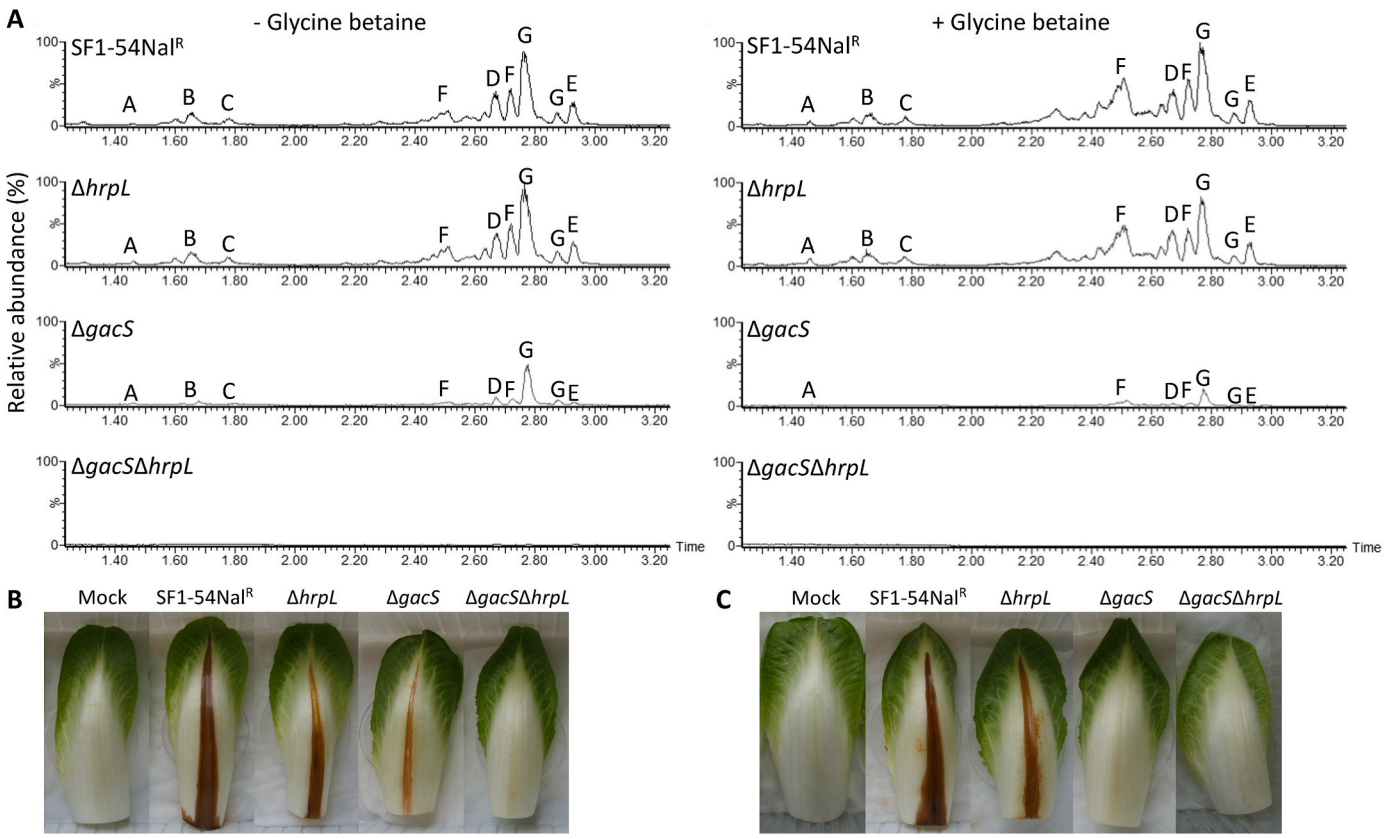

Fig. 5. Lipopeptide production by *Pseudomonas cichorii* strain SF1-54 and mutants. (A) Chromatograms of the semipurified extracts of the culture filtrates obtained from *P. cichorii* strains grown in SRM_{AF} medium with or without glycine betaine. Y axes of LC-MS traces are linked at the same scale for comparison of lipopeptide production. Seven bioactive compounds, which are indicated with a letter, in the semipurified culture extracts were identified by electrospray ionization mass spectrometry analysis. A, uncharacterized lipopeptidic compound. B and C, pseudomycin-like compounds. D and E, cichofactins. F and G, cichopeptins. *P. cichorii* strains are indicated. (B and C) Phytotoxicity of culture filtrates obtained from *P. cichorii* strains grown in SRM_{AF} medium (B) or SRM_{AF} medium containing glycine betaine (C).

pseudomonads, including production of lipopeptides [2,3,6–8]. Strikingly, our data show that the *gacS* mutant of *P. cichorii* SF1-54 did not completely abolish the production of cichofactins and cichopeptins, which contribute to virulence of *P. cichorii* SF1-54 [25,27]. Comparison of lipopeptide production between the *gacS* mutant and the *gacS hrpL* mutant indicates that HrpL is involved in regulating the production of cichofactins and cichopeptins (Figs. 3–5), but activation of lipopeptide production was much weaker by HrpL than by Gac regulon (Fig. 9). Previous studies have already demonstrated that HrpL can also regulate non-T3SS virulence factors in plant pathogenic pseudomonads [41,42]. Thus, the regulation of lipopeptide production seems to be more sophisticated in *P. cichorii* SF1-54 than in other pseudomonads.

We previously demonstrated that production of cichopeptin is linked to glycine betaine metabolism in *P. cichorii* SF1-54 [27]. Based on the results of this study, we speculate that GacS might be involved in sensing plant signals or glycine betaine in the environment and subsequently activating lipopeptide production. Moreover, our results suggest that an additional unknown sensor may also be involved in regulation of lipopeptide production in *P. cichorii* SF1-54 in addition to GacS since the *gacS* mutant was not completely impaired in lipopeptide production. For instance, several LuxR-type regulator genes are located in the flanking regions of cichofactin (*cifR1*, *cifR2*) and cichopeptin biosynthetic gene clusters (*cipR1*, *cipR2*, *cipR3*) in *P. cichorii* SF1-54 [43]. LuxR-type regulators have been shown to be involved in regulation of lipopeptide synthesis in pseudomonads [8,44–46]. In *P. syringae* pv. *syringae* B301D and B728a, three LuxR-type regulators which are SalA, SyrF, and SyrG were shown to activate production of syringomycin and syringopeptin [8,46]. Interestingly, only CipR1 and CipR3 of *P. cichorii* SF1-54 are deduced homologs to SalA and SyrG, respectively [43], but no SyrF homolog was found. Moreover, a putative Rsm binding motif [3] was found in the *cipR1* gene but not in the *cipR3* gene. Thus, we suggest that *cipR1* expression could be under regulation by Gac/Rsm system and *cipR3* expression might be activated by CipR1. A deduced model is proposed based on the results of this study and *P. syringae* [8,46] (Fig. 9). Further investigation is necessary to clarify the regulatory systems involved in lipopeptide biosynthesis in *P. cichorii* SF1-54.

Our data on lipopeptide production suggest that *hrpL* expression in *P. cichorii* is dependent on nutrient levels and osmolarity in the environment. It is well known that *hrpL* expression is highly induced in *P. syringae* under nutrient-limited and low osmotic conditions, while in nutrient-rich and high osmotic environments, *hrpL* expression is low [41,47,48]. Our results clearly show that HrpL slightly up-regulates lipopeptide production in nutrient-limited and low osmotic conditions such as SRM_{AF} medium without supplementation of glycine betaine, but the up-regulation of lipopeptide production by HrpL was reduced while glycine betaine was supplemented into SRM_{AF} medium. Although glycine betaine as a precursor of glycine biosynthesis is linked to cichopeptin formation by *P. cichorii* SF1-54 [27], addition of glycine betaine may result in elevated osmolarity to suppress the level of HrpL activation and subsequent lipopeptide production in *P. cichorii* SF1-54 (Fig. 9). As mentioned by Mansfield [49], parasitism is a mode of microbial nutrition and plant pathogenic pseudomonads must establish an intimate nutritional relationship with the living cells of their hosts during the early biotrophic phase. Moreover, water is more limited in the apoplastic leaf sites than on the epiphytic leaf sites [50]. Accordingly, we suggest that *P. cichorii* uses a sophisticated regulatory network

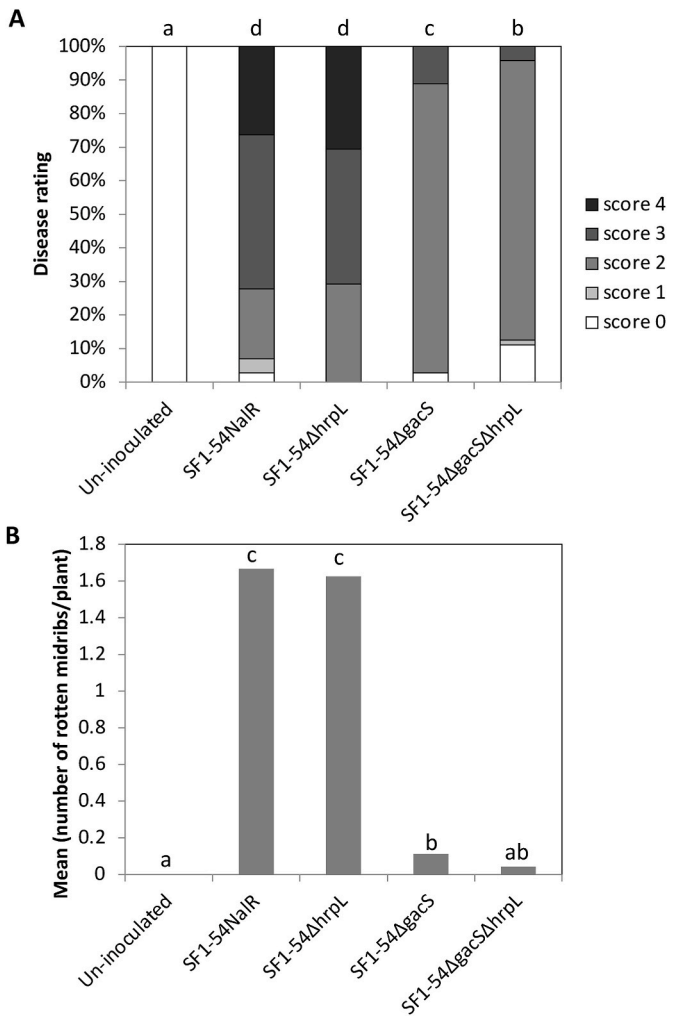

Fig. 6. Virulence of *P. cichorii* SF1-54Nal^R and its mutants on greenhouse grown butterhead lettuce. Plants were inoculated with suspensions of *P. cichorii* strains (1×10^6 CFU/ml) after head formation of lettuce. Virulence is represented as disease severity (A) and mean number of diseased midribs (B). Tap water was used as the un-inoculated control. Bars indicated with the same letter are not statistically different based on nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$).

to efficiently respond to environmental stimuli, produce virulence factors (such as lipopeptides and the T3SS) and avoid trade-offs of energy consuming pathways.

4.2. Contribution of both GacS-regulated lipopeptides and the T3SS to virulence of P. cichorii

We show that the *hrpL* mutant is as virulent as the wild type on lettuce regarding the mean disease scores, numbers of induced rotten midribs and *in planta* bacterial growth (Figs. 6 and 7). To confirm these data, a *hrcC*-deletion mutant of *P. cichorii* SF1-54Nal^R was constructed and characterized. The virulence of the *hrcC* mutant on lettuce, was almost identical to that of the *hrpL* mutant (Supplementary Fig. S5). Thus, the results reflect that T3SS alone is neither a pathogenicity determinant nor an important virulence factor for *P. cichorii* SF1-54 on lettuce, a susceptible host. Similarly, the T3SS mutants of *P. syringae* pv. *syringae* B728a, a strain that produced the lipopeptides syringafactin, syringopeptin SP22 and syringomycin (Girard et al., 2020), can cause disease symptoms in its native host bean and grow *in planta* (Deng et al., 2000; Hirano et al., 1999). Given that the T3SS is not a key player for pathogenicity of *P. cichorii* on lettuce, we hypothesized that the GacS/GacA two-component system may control pathogenicity of *P. cichorii*. Interestingly, *P. cichorii* SF1-54Δ*gacS* exhibited different phenotypes from the *gacS/gacA* mutants or the *rsmA*-overexpressing strains of *P. syringae* [51,52] although *P. cichorii* phylogenetically belongs to the *P. syringae* group [17]. Deficiency in GacS significantly reduced symptom development of *P. cichorii* SF1-54 on lettuce (Fig. 6) but not *in planta* growth of *P. cichorii* SF1-54 (Fig. 7). Our results are in accordance with previous findings demonstrating that the GacS/GacA two-component system is not required for *in planta* growth of *P. syringae* [5,11,53]. Furthermore, our data on lipopeptide production and pathogenic properties clearly provide evidence for the importance of GacS-regulated cichofactins and cichopeptins for virulence of *P. cichorii* SF1-54 on lettuce.

The contribution of the Gac regulon and the T3SS to virulence of *P. cichorii* could be clearly deciphered by pathogenicity assays on plants of different disease tolerance. On a disease-tolerant host (chicory), *P. cichorii* SF1-54 mutants deficient in either GacS or HrpL were virulent but *P. cichorii* SF1-54Δ*gacS*Δ*hrpL* lost its virulence, which is also reflected in the significantly lower populations in chicory leaves compared with the wild type and the single mutants. Thus, GacS-regulated lipopeptides and the T3SS together determine the virulence of *P. cichorii* SF1-54 on a disease-tolerant host. Furthermore, in a disease susceptible host such as lettuce, the role of the T3SS was not pronounced. The function of the T3SS in lettuce-*P. cichorii* interactions is masked by phytotoxic metabolites because *P. cichorii* SF1-54 produces phytotoxic cichopeptins during the early infection stages [27]. Moreover, the results of pathogenicity assays and lipopeptide production show that the T3SS, especially the HrpL regulon, of *P. cichorii* SF1-54 is not under regulation by the GacS/GacA two-component system. In *P. syringae*, it is clear that HrpL controls expression of the T3SS [15,54] but HrpL is not always under the Gac/Rsm regulation. In the model strain *P. syringae* pv. *tomato* DC3000, GacA repressed T3SS expression [11]. By contrast, the Gac regulon controls expression of pathogenicity determinants of *P. syringae* pv. *tabaci* including the T3SS [53,55]. On the other hand, the T3SS is not under the Gac/Rsm regulation in the lipopeptide producing strains *P. syringae* pv. *syringae* B728a and *P. syringae* pv. *syringae* B301D and the phaseolotoxin producing strain *P. syringae* pv. *phaseolicola* 1448A [7,8]. Our results reveal that the regulatory network of *P. cichorii* SF1-54 (Fig. 9) may be similar to that of the lipopeptide producers *P. syringae* pv. *syringae* strains B728a and B301D [8].

It should be noticed that the *gacS* mutation greatly but not completely abolish lipopeptide production by *P. cichorii* SF1-54. A mutant of *P. cichorii* SF1-54 fully impaired in production of all lipopeptide compounds will be important to clarify contribution of the whole lipopeptides to plant virulence of *P. cichorii* SF1-54. We suggest to knock out a gene encoding a Sfp-type 4-phosphopantetheinyl transferase in the genome of *P. cichorii* SF1-54 to achieve this goal. Sfp-type 4-phosphopantetheinyl transferases, which are responsible for catalyzing conversion of non-ribosomal peptide synthetase from apo enzymes to holo enzymes, are necessary for lipopeptide production [56,57]. Moreover, it proved to be very challenging to construct a correct cichopeptin mutant of *P. cichorii* SF1-54 [27], and several attempts failed to obtain a cichofactin and cichopeptin double mutant during the period of this study. Thus, it seems unlikely to knock out all lipopeptide biosynthetic gene clusters of *P. cichorii* SF1-54. Since the genome of *P. cichorii* SF1-54 carries a single Sfp-type 4-phosphopantetheinyl transferase, it would be a good candidate for further study.

P. cichorii is a broad-host-range phytopathogenic bacterium, which is reflected in the genetic structure of its pathogenicity island. In *P. syringae* strains, a pathogenicity island has a tripartite mosaic structure which consists of a central *hrp/hrc* cluster with effector loci at 5′- and 3′-end [58], and several effectors are host-range determinants [59,60]. By contrast, the T3SS gene clusters in *P. cichorii* SF1-54 and other *P. cichorii* strains form a S-PAI without effector loci [18,38], and no other gene encoding a protein similar to known effectors of *P. syringae* could be found in the SF1-54 genome. Previous studies also demonstrated that

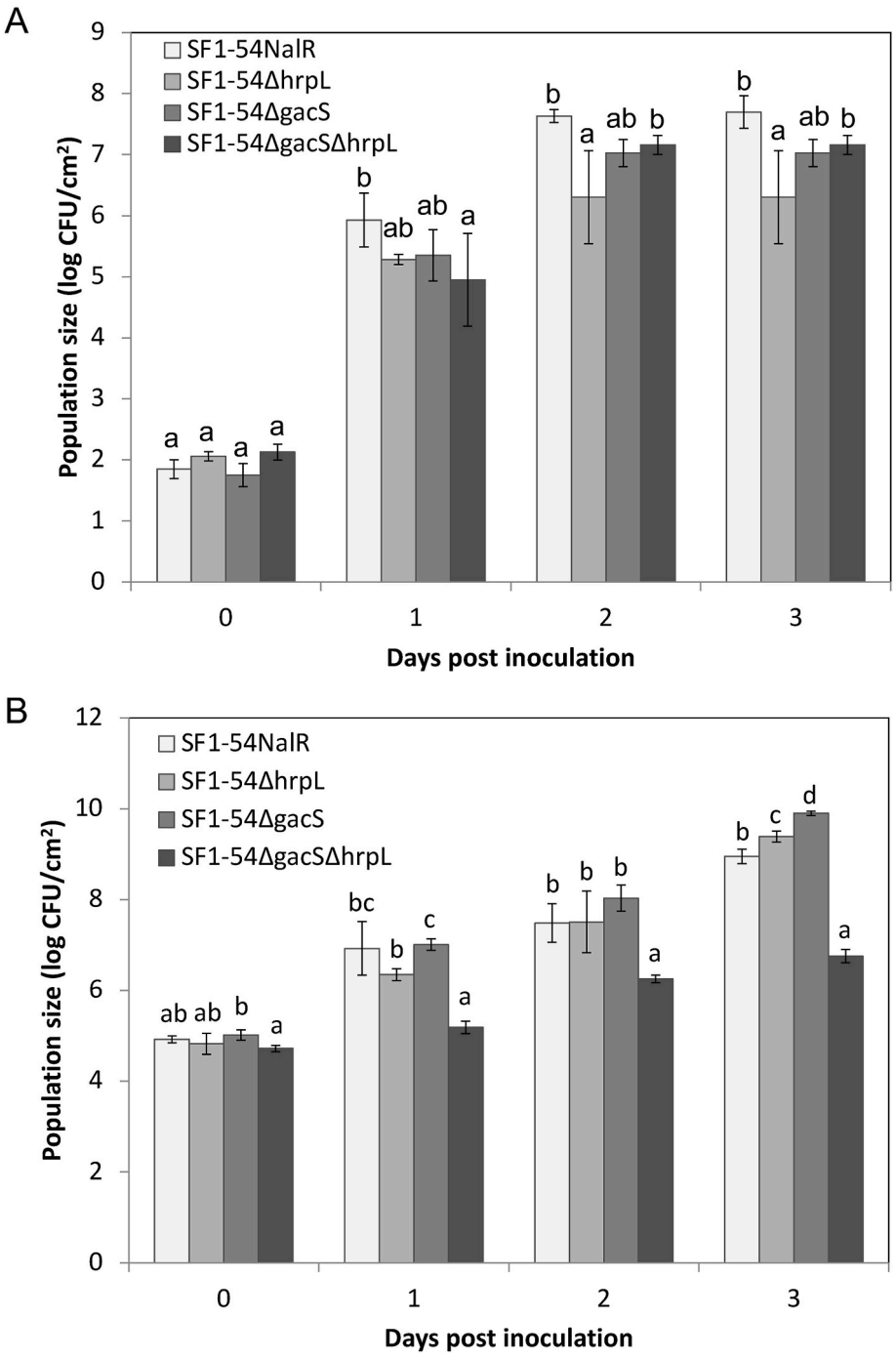

Fig. 7. Population sizes of *P. cichorii* SF1-54Nal^R and its mutants in midribs of lettuce (A) and chicory (B). Values represent the mean ± standard deviation of three independent experiments. Bars indicated with the same letter are not statistically different based on Fisher's Least Significant Difference ($P < 0.05$).

no homologs of *avrPph3* and *avrPphE*, two *avr* genes of *P. syringae* pv. *phaseolicola*, can be detected in *P. cichorii* NCPPB 2379 [61,62]. This unique phenomenon may explain why *P. cichorii* can cause diseases on various host plants since *P. cichorii* does not secrete targets of resistance proteins. Furthermore, the T3SS cluster in the SF1-54 genome has *avrE1* and two harpin-encoding genes (*hrpW* and *hrpZ*) but no *hopA1*, indicating HopA1 is not a conserved T3SS effector of *P. cichorii*. The two harpins are involved in virulence of *P. cichorii* on eggplants [18,19]. Recently, AvrE1 was shown to be essential for virulence of *P. cichorii* JBC1 on cabbage and tomato, especially during the early infection stage [22]. In *P. syringae* pv. *tomato* DC3000, AvrE has been identified as a suppressor of salicylate-dependent basal defense [63,64]. Recently, AvrE family effectors were proven to directly function as water- and solute-permeable channels, which are capable of increasing *in planta* water permeability [65]. It was also proposed that plant pathogenic bacteria may use AvrE channels to create a water- and nutrient-rich apoplast within the infected plant tissues where plant pathogenic bacteria multiply [65]. We suggest that AvrE1 of *P. cichorii* may contribute to creating favorable conditions in infected plant tissues for bacterial multiplication and pathogenesis, but this remains to be investigated. It may explain, however, the lower colonization of the *hrpL* mutant in lettuce plants.

In addition to the T3SS, *P. cichorii* SPC9018 requires iron acquisition to cause symptoms on chicory, eggplant, and sweet pepper but not on

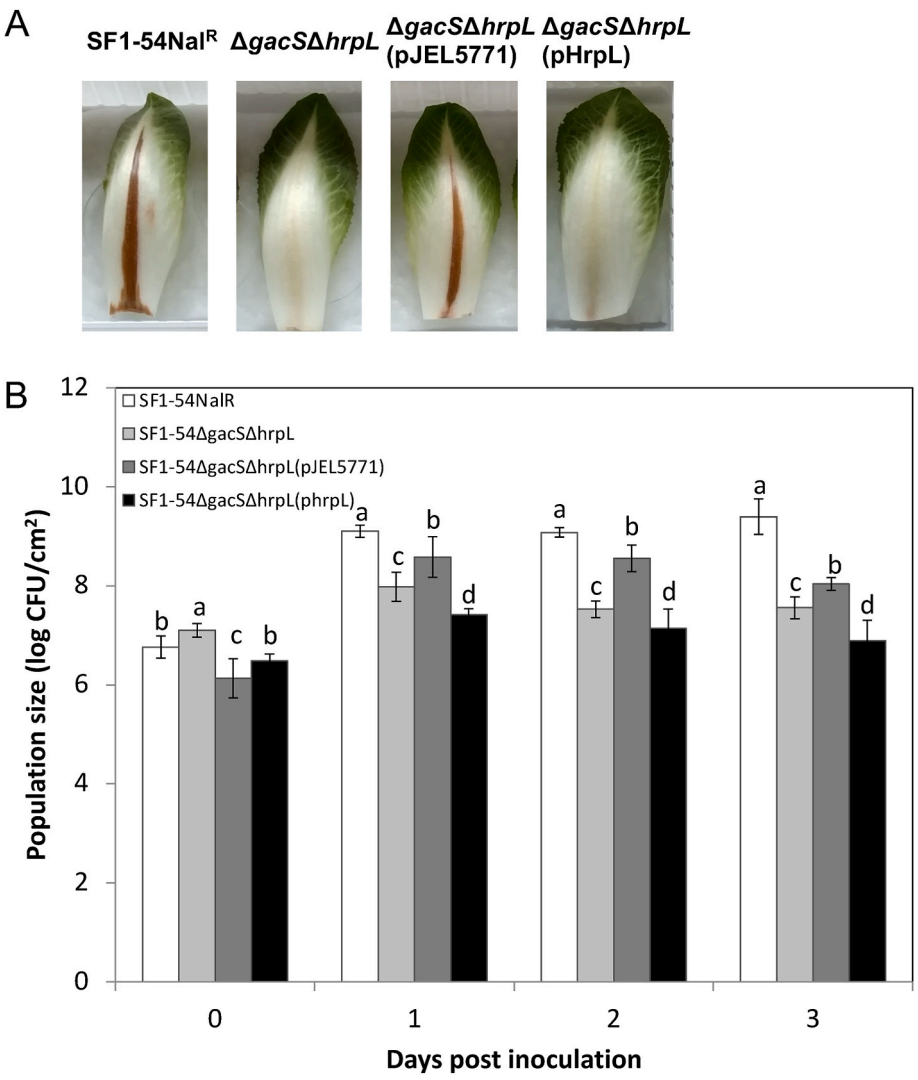

Fig. 8. Complementation of *gacS* or *hrpL* mutation in the *gacS* and *hrpL* double mutant of *P. cichorii*. Chicory leaves were injected with bacterial suspensions of *P. cichorii* strains. *P. cichorii* strains are indicated. Photographs were taken at one day post inoculation (A). Each treatment has been repeated three times and a picture representative of each treatment is shown. (B) Population sizes of *P. cichorii* SF1-54Nal^R, the double mutant, and the complemented strains in midribs of chicory. Values represent the mean ± standard deviation of three independent experiments. Bars indicated with the same letter are not statistically different based on nonparametric Kruskal-Wallis and Mann-Whitney comparisons ($P < 0.05$).

lettuce [18–21]. Like other fluorescent pseudomonads, *P. cichorii* produces pyoverdine to effectively acquire iron from the environments [20,21]. In select phytotoxin-producing strains of *P. syringae*, production of pyoverdine is activated by the Gac/Rsm regulation [7]. Likewise, production of pyoverdine by *P. cichorii* SF1-54 is under GacS control (Fig. 9, Supplementary Fig. S1). Our results again support that pyoverdine-mediated iron acquisition contributes to virulence of *P. cichorii* on chicory but not on lettuce inasmuch as *P. cichorii* SF1-54Δ*gacS*Δ*hrpL* was avirulent on chicory but exhibited its virulence on lettuce.

Hojo et al. [18] suggested that *P. cichorii*-lettuce interaction shows characteristics of a more necrotrophic interaction. Our results are in full agreement with their investigation. The interactions between lettuce/chicory and *P. cichorii* SF1-54 showed similarities with the necrotrophic pathogen *Dickeya dadantii*-plant interactions [66]. Necrotrophic pathogens are 'brute force' pathogens, killing plant cells upon tissue colonization by producing pathogenicity factors such as toxins or plant cell wall degrading enzymes. The T3SS and HrpL-regulated genes play a minor role during *D. dadantii* infection [67], and their function is masked by other virulence factors like cell wall degrading enzymes [66]. However, the T3SS of *D. dadantii* is required for full pathogenicity and initial multiplication within the disease-tolerant (stringent) host plant but not for symptom development [66]. Our results provide evidence that *P. cichorii* shows similar characteristics.

4.3. Negative autogenous control of HrpL in P. cichorii

We failed to complement *hrpL* in the mutant SF1-54Δ*gacS*Δ*hrpL* by plasmid-borne expression of *hrpL* to restore production of lipopeptides and virulence on chicory to levels observed for the *gacS* mutant. We suggest that the expression of *hrpL* on pHrpL could be not sufficient to activate the T3SS in *P. cichorii* SF1-54. A similar phenomenon has been observed in *P. syringae* pv. *phaseolicola* NPS3121 and *P. syringae* pv. *tomato* DC3000. Plasmid-borne *hrpL* could not restore the expression of *hrpL* in the *hrpL* mutants of these *P. syringae* strains [16,68]. It was reasoned that the *hrpL* expression in *P. syringae* pv. *tomato* DC3000 was negatively regulated by cell density, while this cell-density dependent manner was not mediated by quorum sensing [48]. Moreover, the expression of *hrpL* in *P. syringae* pv. *tomato* DC3000 is regulated by negative autogenous control, which is associated with the RNA polymerase (RNAP)-HrpL complex binding the *hrpL* box in the *hrpJ*

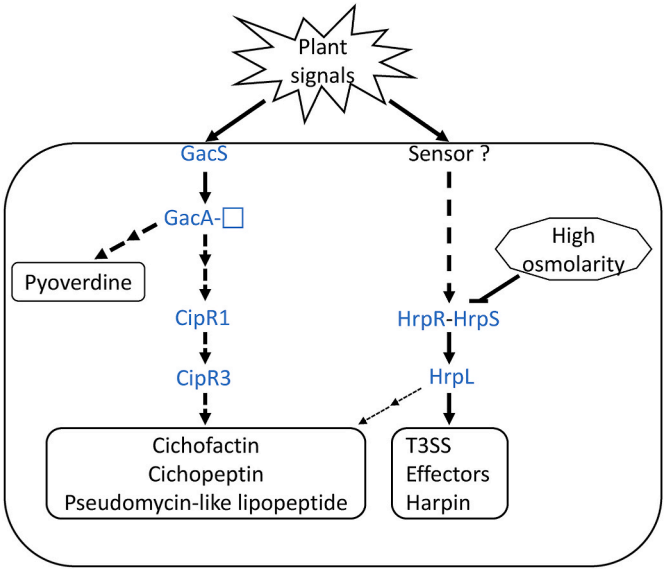

Fig. 9. A Schematic diagram of the putative regulatory network of the GacS-GacA two-component system and the HrpL signal transduction pathway in *Pseudomonas cichorii* SF1-54. GacS is presumably associated with the perception of unknown plant signals and phosphorylates GacA, which might activate CipR1 (a SalA homolog) CipR3 (a SyrG homolog), and the major production of the nonribosomal lipopeptides including cichofactin, cichopeptin, and pseudomycin-like compound (broad arrows). In addition, plant signals also activate the HrpL signal transduction pathway leading to the expression of the type three secretion system (T3SS) and the minor production of nonribosomal lipopeptides (thin arrows). Solid arrows indicate direct regulation, and dashed arrows indicate proposed regulatory pathways. Bars indicate repression.

promoter, to fine-tune T3SS expression [16]. This negative regulatory mechanism resulted in repressed expression of *hrpL* on plasmids [16]. Self-repression of HrpL was additionally associated with occupation of the HrpRS binding region in the *hrpL* promoter by the RNAP-HrpL complex [40]. In our test on chicory, a high-density inoculum of *P. cichorii* was used. *P. cichorii* SF1-54, like three reference strains of *P. syringae*, has a *hrp* box and a HrpS binding site in the intergenic region between *hrpJ* and *hrpL*. Together, it would be reasonable to relate the two mechanisms to the unsuccess of *hrpL* complementation in our study.

In conclusion, both the GacS/GacA two-component system and T3SS are required for virulence of *P. cichorii* SF1-54 during plant pathogenesis. Our previous study showed that *P. cichorii* SF1-54 produces phytotoxic cichopeptins at early infection stages [27], indicating that cichopeptins are possibly involved in initial induction of host cell death by *P. cichorii* SF1-54. However, GacS-regulated cichopeptin is not the only necrosis-inducing factor. The T3SS is also involved in symptom development. Intriguingly, the double mutant of *P. cichorii* SF1-54 deficient in both GacS and T3SS is still pathogenic on lettuce, indicating that there are other unknown factors that induce necrosis in this host plant. Apparently, these unknown virulence factors are not under GacS and HrpL regulation. Thus, further in-depth studies are necessary to identify the pathogenicity determinant(s) and to dissect the regulatory network of virulence in *P. cichorii*, a unique phytopathogenic pseudomonad.

CRediT authorship contribution statement

Chien-Jui Huang: Writing – original draft, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Ellen Pauwelyn:** Writing – original draft, Investigation, Conceptualization. **Marc Ongena:** Writing – review & editing, Formal analysis. **Peter Bleyaert:** Resources. **Monica Höfte:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2024.104249.

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